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(54) Title: A METHOD OF TREATING A MALIGNANCY IN A SUBJECT AND A PHARMACEUTICAL COMPOSITION
FOR USE IN SAME

(57) Abstract: There is disclosed a method of killing abnormal cells such as malignant cells including melanoma cells, using a virus recognising at least one of a cell adhesion molecule and a complement regulatory protein. The virus may be a member of the *Picornaviridae* family. Coxsackie A-group viruses have been found to be particularly suitable. The cell adhesion molecule is desirably a member of the immunoglobulin (Ig) superfamily. Typically, the complement regulatory protein will be DAF.

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A METHOD OF TREATING A MALIGNANCY IN A SUBJECT AND A
PHARMACEUTICAL COMPOSITION FOR USE IN SAME

Field Of The Invention

5 The present invention relates to the killing of abnormal cells utilising a virus. There is also described a method of screening cells to ascertain whether they are susceptible to treatment with the virus, as well as pharmaceutical compositions incorporating the virus. The invention finds veterinary use as well as broad application in the human medical field.

10 Background Of The Invention

 Melanoma is a leading cause of morbidity in the human population. Australia has the highest rate of melanoma in the world. Melanoma is an aggressive skin cancer and is the third most common cancer in Australia for both men and women. It is predicted that one in thirty Australians have a form of melanoma resulting in the death of more than
15 one thousand people per year in that country alone. When detected early most forms of melanoma can be effectively treated. However, the control of more advanced forms is less successful and an area of intensive research. A major goal in this area of research is the identification of molecules that are differentially expressed in benign and malignant melanocytic tumours that can be used for diagnosis and as targets for anti-cancer
20 therapies (Kageshita T. et al; 1993).

 Intercellular adhesion molecule-1 (ICAM-1), a crucial molecule in cellular inflammatory interactions, is an accepted melanoma progression antigen. Surface-

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expression of ICAM-1 on melanomas has been highly correlated with malignant melanoma progression (Kraus A. et al; 1997 and Morandini R. et al; 1998).

ICAM-1 is a member of the immunoglobulin (Ig) superfamily and a counter receptor for the integrin leucocyte function antigen-1 (LFA-1/CD11a) and Mac-
5 (CD11b), and is a cellular attachment molecule for 90% of human rhinoviruses (Stuanton D.E., et al; 1989). In addition, ICAM-1 plays an important role in the pathogenesis of not only rhinovirus infection, but also in *Plasmodium falciparum* infection and in the exacerbations of asthma, chronic bronchitis and cystic fibrosis. Recently, complement regulatory proteins have been reported to be up-regulated on the
10 surface of malignant melanomas, in particular decay-accelerating factor known as DAF (Cheung NK et al; 1998).

Viruses capable of inducing lysis of malignant cells through their replication process are known as oncolytic viruses and trials using oncolytic viruses to treat malignancies have been performed (Nemunaitis J; 1999). Most oncolytic viruses require
15 proliferation in the same species or cell lineage. Infection of a cell by a virus involves attachment and uptake into the cell which leads to or is coincidental with uncoating of the viral capsid, and subsequently replication within the cell (Fenner F., et al. The Biology of Animal Viruses. Academic Press. New York, 1974 Second Ed.)

Oncolytic viruses assessed for capacity to kill cancer cells have included the
20 adenovirus subtype Egypt 101 virus which showed oncolytic activity in the HeLa uterine/cervix cancer cell line, mumps virus for treatment of gastric carcinoma, uterine carcinoma and cutaneous carcinoma, Newcastle Disease Virus (NDV), influenza virus for treatment of ovarian cancer, and adenovirus for treatment of for instance, cervical

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carcinoma (Nemunaitis J; 1999). Other reports have indicated that adenoviruses and attenuated poliovirus recombinants may have use in the treatment of malignant glioma cells (Alemany R., et al 1999; Andreansky S.S., 1996), and that reovirus shows lytic capability in human U87 glioblastoma cells and NIH-3T3 cells with an activated Ras signalling pathway (Coffey M.C, et al, 1998; Strong J.E. et al, 1998).

In addition, a vaccinia oncolysate has been used in clinical trials to treat melanoma (Stage II) patients (Nemunaitis J., 1999). Modified, non-neurovirulent Herpes simplex viruses (HSV) have also been reported as showing promise for the treatment of brain tumours including intracranial melanoma, and subcutaneous human melanoma (Randazzo B.R., 1997), while adenovirus infection has been reported to enhance killing of melanoma cells by the plant mitotoxin, saporin (Satyamoorthy K., 1997).

The receptor on target cells recognised by adenovirus differs for different adenovirus types. That is, adenovirus subgroups A, C, D, E and F for instance recognise the CAR receptor while Adenovirus type 5 (subgroup C), Adenovirus type 2 (subgroup C) and Adenovirus type 9 (subgroup D) recognise major histocompatibility class II molecule, $\alpha_m\beta_2$ and α_v integrins, respectively. The CAR receptor is known to be expressed on melanoma cell lines (Hemmi S., et al, 1998). Heparan sulfate is recognised by Herpes simplex types 1 and 2 and human herpes virus 7, Adeno-associated virus type 2. The receptor for human Herpesvirus 7 is CD4 while Epstein-Barr virus recognises complement receptor Cr2 (CD21). Poliovirus type 1 and 2 recognise poliovirus receptor (Pvr) for cell adhesion while reovirus recognises sialic acid. Influenza A and B virus recognise the sialic acid N-acetyl neuraminic acid for cell adhesion. In contrast, influenza type C virus recognises the sialic acid 9-O-acetyl

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neuraminic acid. Vaccina virus recognises both epidermal growth factor receptor and heparan sulfate. Coxsackievirus A13, A15, A18 and A21 recognise ICAM-1 and the complement regulatory protein DAF (CD55) (see eg. Shafren D.R., et al 1997). DAF is also recognised by Enterovirus 70. See for instance Flint SJ, et al (2000) Principles of Virology:molecular biology, pathogenesis and control. ASM Press, Washington.

Metastatic tumour spread is a pathological process associated with a series of adhesion/de-adhesion events coupled with regulated tissue degradation. It is known that adhesion to and migration through the extracellular matrix is essential for tumour invasion. The largest family of extracellular adhesion molecules is the integrin family (Marshall J.F. and Hart I.R., 1996) and members of the $\alpha_v\beta$ group of integrins have been shown to be expressed on a variety of cell types. For instance $\alpha_v\beta_1$ is expressed on neuroblastoma, melanoma and osteosarcoma cells, $\alpha_v\beta_3$ is expressed on melanoma, glioblastoma and renal carcinoma cells, and $\alpha_v\beta_5$ is expressed on melanoma cells as is $\alpha_v\beta_8$ (Marshall J.F. and Hart I.R., 1996).

Despite progress being made in the treatment of malignancies, the treatment of cancer including melanoma presents a major challenge for research and there remains the need for alternatives to existing therapy approaches.

Summary Of The Invention

The present invention stems from the surprising finding of significant killing of abnormal cells can be achieved with the use of a virus and the recognition/interaction of cell expressed markers utilised by the virus for infectivity of the cells.

In one aspect there is provided a method of treating abnormal cells in a mammal comprising administering to the mammal an effective amount of a virus capable of

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infecting the abnormal cells whereby death of the cells is caused and which recognises at least one of a cell adhesion molecule of the immunoglobulin (Ig) superfamily and a complement regulatory protein for infectivity of the abnormal cells.

The term "abnormal cells" for the purpose of the present invention is to be taken in a broadest sense to include malignant cells, the cells of any abnormal growth and any cells having abnormal upregulated expression of at least one of the cell adhesion molecule and the complement regulatory protein relative to corresponding normal cells of the same cell type expressing their normal phenotype, whether the cells are cancer cells or not and whether the cells proliferate at an abnormal rate or not. Accordingly, the term encompasses pre-neoplastic and neoplastic cells, and non-cancer cells that may or may not ultimately develop into cancer cells. An abnormal growth may for instance be a benign or malignant tumour. Typically, the abnormal cells will be malignant cells and usually melanoma cells.

Generally, the expression of at least one of the cell adhesion molecule and the complement regulatory protein will be upregulated compared to surrounding tissue in which the abnormal cells are found.

Hence, the virus will typically preferentially infect the abnormal cells due to the greater likelihood of contacting at least one of the cell adhesion molecule and complement regulatory protein on those cells. As such the virus may be used to effectively target the abnormal cells.

In another aspect of the invention there is provided a method of treating melanoma in a mammal comprising administering to the mammal an effective amount of a virus capable of infecting melanoma cells whereby death of the cells is caused and wherein the

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virus recognises at least one of a cell adhesion molecule and a complement regulatory protein for infectivity of the melanoma cells.

The virus may also be used to screen cells to ascertain for instance whether the virus may be suitable for treating the patient from which the cells were obtained or
5 whether a different treatment protocol not involving the virus may be more beneficial to the mammal. Conversely, different viruses may be screened using samples of cells taken from the patient in order to select the most appropriate virus for treating the mammal.

Accordingly, in another aspect of the invention there is provided a method of screening abnormal cells for determining whether the cells are susceptible to viral
10 induced cell death, comprising the steps of:

- (a) providing the abnormal cells;
- (b) adding to the cells an effective amount of a virus which recognises at least one of a cell adhesion molecule of the immunoglobulin (Ig) superfamily and a complement regulatory protein for infectivity of the abnormal cells;
- 15 (c) incubating the abnormal cells in the presence of the virus for a period of time; and
- (d) determining whether the virus has infected and caused death of at least some of the abnormal cells.

In a further aspect of the present invention there is provided a method of screening
20 melanoma cells for determining whether the cells are susceptible to viral induced cell death, comprising the steps of:

- (a) providing the melanoma cells;
- (b) adding to the melanoma cells an effective amount of a virus which

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recognises at least one of a cell adhesion molecule and a complement regulatory protein for infectivity of the melanoma cells;

(c) incubating the melanoma cells in the presence of the virus for a period of time; and

5 (d) determining whether the virus has infected and caused death of at least some of the melanoma cells.

A virus may be selected for use in a method of the invention by testing whether a given virus is capable of infecting and causing the death of abnormal cells expressing at least one of the cell adhesion molecule and the complement regulatory protein. In
10 particular, the testing may involve screening a number of different viruses by incubating each virus with a sample of the abnormal cells respectively, and determining whether the cells are killed as a result of infection.

Accordingly, in another aspect of the present invention there is provided a method of testing whether a virus is capable of infecting abnormal cells whereby death of the
15 cells is caused and which recognises at least one of a cell adhesion molecule of the immunoglobulin (Ig) superfamily and a complement regulatory protein for infectivity of the abnormal cells.

In a further aspect of the present invention, there is provided a method of testing whether a virus is capable of infecting melanoma cells whereby death of the cells is
20 caused and which recognises at least one of a cell adhesion molecule and a complement regulatory protein for infectivity of the melanoma cells.

In still another aspect of the invention there is provided a method of screening a virus for ability to infect and cause death of abnormal cells, comprising the steps of:

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(a) selecting a virus which recognises at least one of a cell adhesion molecule of the immunoglobulin (Ig) superfamily and a complement regulatory protein for infectivity of the abnormal cells;

(b) incubating the selected said virus with a sample of the abnormal cells for a
5 period of time; and

(c) determining whether the selected said virus causes death of at least some of the abnormal cells.

In another aspect of the present invention there is provided a method of screening a virus for ability to infect and cause death of melanoma cells, comprising the steps of:

10 (a) selecting a virus which recognises at least one of a cell adhesion molecule and a complement regulatory protein for infectivity of the melanoma cells;

(b) incubating the selected said virus with a sample of the melanoma cells for a period of time; and

(c) determining whether the selected said virus causes death of at least some of
15 the melanoma cells.

The method may also comprise the step of comparing the ability of the selected virus to infect and cause the death of the cells with that of another virus subjected to steps (b) and (c) utilising another sample of the cells.

Death of the cells following infection with the virus may result from either lysis of
20 the cells due to intracellular replication of the virus or due to the infection triggering apoptosis most likely as a result of the activation of cellular caspases.

Once lysed, the cytosolic contents of infected cells spills from the ruptured plasma membranes, and antigens capable of eliciting an immune response to the abnormal cells

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may be released. Hence, treatment of abnormal cells in a mammal in accordance with a method of the invention may provide a boost to the immunity of the mammal against the abnormal cells.

Accordingly, in another aspect of the invention there is provided a method of
5 inducing an immune response in a mammal comprising infecting abnormal cells in the mammal with a virus whereby death and lysis of the cells is caused with release of antigens therefrom for generation of said immune response, wherein the virus recognises at least one of a cell adhesion molecule of the immunoglobulin (Ig) superfamily and a complement regulatory protein for infectivity of the abnormal cells.

10 In yet another aspect of the present invention there is provided a method of inducing an immune response in a mammal against melanoma cells, comprising infecting the melanoma cells in the mammal with a virus whereby death and lysis of the cells is caused with release of antigens therefrom for generation of said immune response, wherein the virus recognises at least one of a cell adhesion molecule and a
15 complement regulatory protein for infectivity of the melanoma cells.

Generally, the virus will be provided in the form of a pharmaceutical composition for use in a method of the invention. As such, in a yet further aspect of the invention there is provided a pharmaceutical composition comprising a pharmaceutically acceptable carrier together with a virus capable of infecting abnormal cells whereby
20 death of the cells is caused and which recognises at least one of a cell adhesion molecule of the immunoglobulin (Ig) superfamily and a complement regulatory protein for infectivity of the abnormal cells.

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In still another aspect of the present invention there is provided a pharmaceutical composition comprising a pharmaceutically acceptable carrier together with a virus capable of infecting melanoma cells whereby death of the cells is caused and which recognises at least one of a cell adhesion molecule and a complement regulatory protein
5 for infectivity of the melanoma cells.

In another aspect is provided the use of the pharmaceutical composition in a method of the invention.

In a further aspect of the invention there is provided the use of a virus in the manufacture of a medicament for treating malignant cells, wherein the virus is capable of
10 infecting the abnormal cells whereby death of the cells is caused and which recognises a cell adhesion molecule of the immunoglobulin (Ig) superfamily for infectivity of said abnormal cells.

In another aspect of the present invention there is provided the use of a virus in the manufacture of a medicament for treating melanoma wherein the virus is capable of
15 infecting melanoma cells whereby death of the cells is caused and which recognises at least one of a cell adhesion molecule and a complement regulatory protein for infectivity of the melanoma cells.

In addition, there is provided delivery means for being held against the skin of a mammal for facilitating delivery of the virus to the mammal, and which is impregnated
20 with a pharmaceutical composition of the invention for contact with the skin, when the delivery means is held against said skin of the mammal in use. Generally, the delivery means will be adapted to enable it to hold in position over the skin at the desired site of treatment.

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Preferably, the virus will be capable of binding to or otherwise associating with both the cell adhesion molecule and the complement regulatory protein. The complement regulatory protein will usually form a complex with the cell adhesion molecule or have a close spatial association with the cell adhesion molecule, and
5 enhance the ability of the virus to infect the abnormal cells. Preferably, the complement regulatory protein will be decay-accelerating factor (DAF).

Preferably, the cell adhesion molecule is a member of the immunoglobulin (Ig) superfamily which includes V-CAM-1 and the intercellular adhesion molecules ICAM-1, ICAM-2 and ICAM-3. Preferably, the cell adhesion molecule is ICAM-1.

10 Normally, the virus will be an animal RNA virus and typically, a non-enveloped RNA virus with an icosohedral capsid and a single RNA strand genome.

Preferably, the virus will be a member of the *Picornaviridae* family. Members of the immunoglobulin (Ig) superfamily have a plurality of extracellular domains and the virus will desirably interact with the outermost domain closest to the N-terminus of the
15 immunoglobulin (Ig) superfamily molecule. Preferably, the virus will be from the genus *Enterovirus* and most preferably, the virus will be a Coxsackievirus. Coxsackievirus is a human enterovirus and most enteroviral infections, even with the more virulent members of the group, cause few or no clinical symptoms. CAV21 infection for instance is associated with development of common colds and infantile diarrhoea.

20 Hence, in another aspect of the present invention there is provided a method of treating abnormal cells in a mammal comprising administering to the mammal an effective amount of a Coxsackievirus.

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In a still further aspect of the present invention there is provided a method of treating melanoma in a mammal comprising administering to the mammal an effective amount of a Cocksackievirus.

Typically, the Cocksackievirus will be a Cocksackie A-group virus, and will
5 normally be selected from the group consisting of Cocksackieviruses serotypes 1 to 24 (CAV1-24), and most preferably from CAV13, CAV15, CAV18 and CAV21.

While the virus will usually be a common animal virus the invention is not limited thereto and a recombinant virus engineered to be capable of infecting and causing the death of the abnormal cells, or a virus that has otherwise been modified to enhance its
10 ability to infect the cells and cause the death of the cells post infection, may be utilised. For instance, the virus may be modified to recognise additional cell adhesion molecules such as $\alpha_v\beta_3$, $\alpha_v\beta_5$ or $\alpha_v\beta_6$.

Moreover, the same virus may be administered to the mammal during different treatment courses. Preferably, however, different viruses are used for different treatment
15 courses to avoid or lessen the potential effect of any immune response to the previous virus administered. The virus may for instance be administered topically, intratumourally or systemically to the patient.

The mammal may be any mammal suffering from a malignancy and in need of treatment. Preferably, the mammal will be a human being.

20 A method of the invention may be used as an adjunct to conventional cancer treatment or as a treatment in the absence of other therapeutic treatments. In particular, a method of the invention may be utilised where conventional treatment is not suitable or practical, or in the instance where excision of abnormal cells may leave scarring or

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disfigurement which is unacceptable to the patient, particularly the patient's face such as from their nose or lip. Alternatively, the virus may be administered to the patient prior to and/or immediately after excision of abnormal cells.

Accordingly, the instant methods provide an alternative therapeutic treatment
5 which may be used both following diagnosis of early stage and latter stage malignancy, and which further finds application for killing cells prior to and remaining after surgery.

Using protocols as described herein the skilled addressee will be able to readily select a suitable virus for use in the methods of the invention, and determine which abnormal cells are susceptible to infection leading to the death of the cells. The
10 abnormal cells may for instance be prostate cancer cells, breast cancer cells, stomach cancer cells, gastric carcinoma cells, colon cancer cells, colorectal cancer cells, glioma cancer cells, skin cancer cells or other malignant cells.

A method of the invention is particularly suitable for treating a malignancy of the skin or a malignancy that has spread from the skin such as melanoma.

15 Unless the context clearly requires otherwise, throughout the description and the claims, the words 'comprise', 'comprising', and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

The invention will now hereinafter be further described with reference to a number
20 of non-limiting preferred embodiments.

Brief Description Of The Accompanying Drawings

Figure 1 shows immunoperoxidase staining of surface ICAM-1 expression on melanoma cells. ICAM-1 expression (white arrows) is indicated by dark cell staining;

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Figure 2 shows relative levels of ICAM-1 and DAF expression by the melanoma cell lines Sk-Mel-28 and ME4405;

Figure 3 indicates lytic infection of two human melanoma cells lines by Cocksackievirus A21 at different time intervals post infection;

5 Figure 4 indicates lytic infection of human melanoma cells from a primary melanoma induced in a nude mouse with various doses of Cocksackievirus A21;

Figure 5 indicates lytic infection of preparations of suspension and adherent primary malignant cells from a chest wall melanoma by Cocksackievirus A21 at 20 hours post infection;

10 Figure 6 (A) indicates lytic infection of six human melanoma cell lines by Cocksackievirus A21 at twenty-three hours post infection; (B) indicates results of flow cytometric analysis of DAF (dark line) and ICAM-1 (lighter line) on the surface of human melanoma cells;

Figure 7 indicates lytic infection of different tumour cell lines by representative
15 human enteroviruses;

Figure 8 indicates lytic infection of a human melanoma biopsy from lymph node by human enteroviruses Cocksackievirus A21 and B3;

Figure 9 indicates lytic infection of prostate cancer cells by selected Cocksackievirus;

20 Figure 10 shows the capacity of CAV21 and CAV15 to specifically lytically destroy melanoma cells without infecting non-melanoma cells;

Figure 11 indicates subcutaneous administration of CAV21 infected cells to NOD-SCID mice inhibits human melanoma tumour formation;

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Figure 12 is a graph showing results of intratumoural treatment of preformed Sk-Mel-28 melanoma with CAV21;

Figure 13 is a graph showing results of intratumoural treatment of preformed Sk-Mel-28 melanoma with CAV15;

5 Figure 14 shows Sk-Mel-28 tumours 35 days post inoculation with PBS (left tumour) and CAV15 (right tumour); and

Figure 15 is a graph showing the effect of intratumoural treatment of preformed ME4405 melanoma with CAV21.

Detailed Description of Preferred Embodiments of the Invention

10 To determine whether a virus is capable of infecting and causing death of cells of a tumour, a biopsy may be taken from the tumour and a preparation of cells prepared using conventional techniques prior to: (i) confirming virus receptor cell surface expression and (ii) challenging the cells with the virus and monitoring the cells for infection and cell death over a predetermined incubation period, typically about 2 days although this may
15 vary depending on the virus used. A number of viruses may be screened in this way simultaneously utilising different aliquot's of the prepared malignant cells, the virus showing the greater degree of infectivity and cell death may then be selected for administration to the subject from whom the biopsy was taken. Similarly, different malignant cell preparations from biopsies taken from different sources may be employed
20 in an assay using a specific virus. The biopsies may be taken from different sites of a single individual or from a number of individuals.

A virus used in a method as described herein will desirably cause few or only minor clinical symptoms in the recipient. Such viruses are readily obtainable from

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commercial sources well known to the skilled addressee and can be screened for their effectiveness in the instant methods in the manner described above. Desirably, the virus will normally be selected from Cocksackie A-group viruses. CAV21 is preferred and in particular CAV21 (Kuykendall) (Sickles G.M., Proc. Soc. Exp. Biol. Med. 102:742; Shafren D. et al J. Virol 1997, 71:4736; Hughes et al, J. Gen Virol. 1989, 70:2943; Schmidt, N.J., et al, Proc. Soc. Exp. Biol. Med., 1961, 107:63. CAV21 (Kuykendall) is available from the American Type Culture Collection (ATCC) 10801 University Boulevard, Manassas, Virginia 20110-2209, United States of America under Accession No. VR-850.

10 For the purpose of simply screening a given virus to ascertain whether it is capable of infecting and causing the death of malignant cells, malignant cell lines may be used for this purpose rather than primary malignant cells isolated from a biopsy.

Virus that recognises at least one of ICAM-1 and the complement regulatory protein DAF will typically be used. Besides being expressed on melanoma cells (Cheung N.K. et al 1998), DAF has also been shown to have upregulated expression on colonic adenocarcinoma cells *in situ* and on the human colonic adenocarcinoma cell line HT29. The expression of DAF has been postulated to promote resistance of the cells to complement mediated damage and so represents a possible mechanism of tumour escape (Björge L., et al; 1996).

20 Upregulated expression of ICAM-1 has been reported in a variety of malignant cell types including gastric carcinoma and adenoma cells (Nasu R., 1996; and Koyama S., 1992), prostate cancer cells (Rokhlin O.W., and Cohen M.B., 1995), and human breast cancer cells (Sgagius M.K., 1996). Studies have also shown that V-CAM1 is expressed

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with ICAM-1 on breast cancer cells (Regidor P.A., et al; 1998). In addition, ICAM-1 is known to be expressed on medullary carcinoma cells (Bacuss S.S. et al; 1994), myeloma cells (Maloney D.G. et al; 1999) and thyroid carcinoma cells. ICAM-1 positive staining has also been reported in primary tumours such as papillary adenocarcinoma, and
5 metastatic tumours from brain, liver and the adrenal gland (Fernandez-Real J.M; 1996).

Tumours occurring on the skin such as melanoma are particularly suitable candidates for treatment with the virus. In instances where melanoma has spread to lymph nodes, the lungs or other organs, the virus may be administered to those sites and/or the surrounding tissue as described above during a surgical procedure to expose
10 such sites for treatment.

The selected virus will preferably be injected directly into a number of sites on a malignant tumour in order to maximise the area for potential infection of the tumour by the virus. Normally, tissue surrounding the tumour will be injected or otherwise treated with the virus given the possibility of malignant cells being present in the tissue. If the
15 tumour is not detected until it is relatively advanced, surrounding tissue may be injected with the virus following surgical excision of the tumour itself.

Rather than being injected directly into a malignant tumour, the virus may be administered systemically by intravenous injection into the blood stream of the recipient at a location adjacent to the tumour site for delivery to the tumour. Similarly, the virus
20 may be administered subcutaneously, intraperitoneally or for instance, intramuscularly if deemed appropriate. Generally, however, direct injection into the tumour is preferred given the possibility of the existence of antibodies specific for the virus and thereby the potential decreased efficacy of alternate such modes of virus delivery.

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The virus may also be applied topically to tumours either alone or in combination with direct injection of the virus into the tumour. In this instance, the virus may be applied by way of delivery means for being pressed against the malignant site on the skin to be treated and which is impregnated with a suitable pharmaceutically acceptable carrier for maintaining the integrity of the virus to allow for infection of the malignant cells by the virus. The delivery means may be in the form of for instance, a patch, a pad, a wad, bandaging or the like suitable for localising the virus in the area to be treated. Typically, the delivery means will be a patch provided with an adhesive around an underside perimeter thereof for sticking the patch on the skin and thereby holding the patch in the desired position and the inoculant in contact with the patients skin.

Generally, one or more small incisions will be made into the malignancy and/or surrounding tissue to provide a site of entry for the virus into same.

The carrier medium used for inoculating the recipient with the virus may be a fluid such as physiological saline, or any other conventionally known medium deemed appropriate such as commercially available gels suitable for pharmaceutical use and for administering the virus to the site of treatment.

The inoculant will generally contain from about 1×10^2 to about 1×10^{10} plaque forming units per ml of the inoculant. Preferably, the inoculant will contain greater than about 1×10^5 plaque forming units per ml of inoculant. The amount of inoculant administered to the patient may be readily determined by the attending physician or surgeon in accordance with accepted medical practice taking into account the general condition of the patient, the stage and location of the malignancy together with the overall size and distribution of the area to be treated with the virus. Typically, the

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patient will be treated with an initial dose of the virus and subsequently monitored for a suitable period of time before a decision is made to administer further virus to the patient pending factors such as the response of the patient to the initial administration of the virus and the degree of viral infection and malignant cell death resulting from the initial treatment.

Desirably, an individual will be treated with the virus over a period of time at predetermined intervals. The intervals may be daily or range from 24 hours up to 72 hours or more as determined appropriate in each circumstance. The same or a different virus may be administered each time to avoid or minimise the effect of any immune response to a previously administered virus, and a course of treatment may extend for one to two weeks or more as may be determined by the attending physician.. Most preferably, virus to which the mammal has not previously been exposed or to which the mammal generates a relatively minor immune response as may be determined by standard techniques will be administered.

While readily available known viruses may be suitably employed in a method of the invention, a virus modified or engineered using conventional techniques may also be utilised. For instance, a virus may be modified to employ additional cell adhesion molecules as cell receptors. For example, Cocksackievirus A21 may be modified using site-directed mutagenesis so that the peptide motif "RGD" is expressed on the viral capsid surface as is the case with Cocksackievirus A9 (CAV-9). The RGD motif is recognised by all the α_v integrin heterodimers and this capsid modification may for instance allow the virus to bind the integrin $\alpha_v\beta_3$, a cell adhesion molecule which has been shown to be up-regulated in combination with ICAM-1 on the surface of malignant

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melanoma lesions (Natali P.G.; 1997) leading to enhanced uptake of the virus via interaction with the integrin molecule or subsequent interaction with ICAM-1.

Alternatively, the virus may be modified to recognise a selectin such as E-selectin.

The invention will now be described with reference to a number of examples

5 described below.

Example 1

1.1. Cell Lines

Continuous cultures of Rhabdomyosarcoma expressing ICAM-1 cells (RD-ICAM-1), HeLa-B cells, and human lung fibroblast cells (MRC5) were maintained in
10 Dulbecco's Modified Eagle's Medium (DMEM) and 10% fetal calf serum (FCS). Two melanoma cell lines Sk-Mel-28 and ME4405 were obtained from Dr. Ralph (Department of Biochemistry and Molecular Biology, Monash University, Victoria, Australia) and Dr. Peter Hersey, Cancer Research Department, David Maddison Building Level 4, Royal Newcastle Hospital, Newcastle, New South Wales, Australia, respectively. The cell line
15 Sk-Mel-28 is a metastatic melanoma cell line found to be resistant to chemotherapeutic drugs (56). The melanoma cell culture ME4405 was established from specimens of primary melanoma lesions (69). The two melanoma cell lines were maintained in DMEM containing 10% FCS. Rhabdomyosarcoma cells (RD) a heteroploid human embryonal cell line, and HeLa-B cells an aneuploid cell clone derived from human
20 squamous epithelial cells, were obtained from the Entero-respiratory Laboratory, Fairfield Hospital, Melbourne, Victoria, Australia. RD cells stably transfected with cDNA encoding the immunoglobulin superfamily molecule ICAM-1 providing the RD-

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ICAM-1 cell line have been described elsewhere (Shafren DR, et al; 1997). MRC5 cells, derived from human lung fibroblasts were obtained from Bio-Whittaker, USA.

1.2. Viruses

Strains of CAV21 (Kuykendall strain), CAV15 (G-9) and CVB3 (Nancy) were
5 obtained from Margery Kennett, Entero-respiratory Laboratory, Fairfield Hospital, Melbourne, Victoria, Australia.

1.3. Virus Propagation

RD-ICAM-1 cultures (80-95% confluent) were infected with 10^4 TCID₅₀ (50%
tissue culture infectious dose) of Coxsackievirus A strains according to standard
10 procedures. Infected cells were incubated at 37°C until complete cytopathic effect was observed (within 2 days). Cells were then frozen at -80°C and thawed to release the remaining intracellular virus particles. The virus-containing medium was clarified of cellular debris by centrifugation for 5 min at 1000 x g and stored as 500 µl aliquots at -80°C. CVB3 was propagated in HeLa-B cells in the same manner as described above.

1.4 Monoclonal Antibodies (MAbs)

MAb IH4 which recognises the third SCR of DAF (24) was a gift from Dr. B. Loveland, Austin Research Institute, Melbourne, Victoria, Australia. MAb WEHI-CAM recognises the first domain of ICAM-1 (Berendt AR, et al; 1992) and was provided by Dr. A. Boyd, Walter and Eliza Hall Institute, Melbourne, Victoria, Australia.

1.5. Flow Cytometric Analysis

20 Cells (1×10^6) in 100 µl aliquots were incubated with Mab IH4 or Mab WEHI-CAM diluted in DMEM containing 1% FCS on ice for 30 min. The cells were then washed with 5.0 ml of PBS, pelleted at 1,000 x g for 5 min and resuspended in 100 µl of

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fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Silenus, Melbourne, Australia) diluted in PBS. Following incubation on ice for 30 min the cells were washed and pelleted, and resuspended in PBS for analysis with a FACStar analyser (Becton Dickinson, Sydney, Australia).

5 **1.6. Colourimetric Infectivity Assay**

The stock virus solutions of CAV21 and CAV15 were serially diluted 10-fold in DMEM containing 1% foetal calf serum (FCS). RD-ICAM-1 cell monolayers in 96-well plates were inoculated with 100 µl of serial dilutions of the viruses for 48h at 37°C. To quantitate cell survival, monolayers were incubated with 100µl of a crystal violet-
10 methanol solution (5% w/v crystal violet, 10% v/v methanol, 10% v/v formaldehyde solution in PBS) and washed with distilled water. The plates were read on a multiscan enzyme-linked immunosorbent assay plate reader at a wavelength of 540 nm. Fifty percent endpoint titres were calculated (Reed LJ and Muench HA; 1938) and expressed as 50% tissue culture infectious dose (TCID₅₀) per millilitre. A well was scored positive
15 if absorbance was less than three standard deviations of the no-virus control. The TCID₅₀ for CAV21 was determined to be 2.7×10^4 units per ml while for CAV15, the TCID₅₀ was determined to be 1.6×10^4 units per ml.

1.7. Surface Expression of ICAM-1 and DAF

The relative levels of ICAM-1 and DAF expression on the surface of the
20 melanoma cell lines SK-Mel-28 and ME4405 was determined by flow cytometric analysis. The results are shown in Fig 2.

As can be seen, flow cytometric analysis revealed comparable high level ICAM-1 and DAF expression on the surface of the two melanoma cell lines. A further 6

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melanoma cell lines derived from metastatic melanomas also expressed high levels of ICAM-1 and DAF (data not shown). The finding of high level ICAM-1 expression on all the metastatic melanoma cells tested supports several reports in the literature noting increased levels of ICAM-1 expression *in vivo* correlates with increased metastatic ability (Johnson JP, et al:1988; Kageshita T, et al:1993; Miller BE and Welch DR:1990; Natalie PG, et al:1997).

Example 2

2.1. Infection of Melanoma Cell Lines by CAV21

Monolayers of two culture-adapted melanoma cell lines Miller and MM200 were infected with CAV21 prepared in Example 1 at a multiplicity of infection of 1.0 for 1 hour prior to removal of the inoculum and the cells incubated in culture medium (DMEM containing 1% foetal calf serum and penicillin streptomycin) for 24 hours at 37°C. The results shown in Fig. 3 indicate that CAV21 was able to induce significant changes in the cellular cytopathology of both cell lines as early as five hours post infection (PI) and by nine hours PI almost complete killing of all the melanoma cells.

Example 3

3.1. Infection of Melanoma Cells from Primary Melanoma by CAV21

Cells from a primary melanoma removed from a nude mouse that had been previously subcutaneously inoculated with human melanoma cells from cell line ME 4405 using conventional methods, were highly susceptible to CAV21 infection and

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killing, even at a challenge rate of 0.005 CAV21 particles per melanoma cell as shown in Fig. 4.

Example 4

5 4.1. Infection of Melanoma Cells Isolated From Tissue Biopsy by CAV21

Melanoma cells were isolated from fresh biopsy of a primary chest wall melanoma by the "spilling" technique and by digestion in collagen-trypsin and DNAase. Briefly, cells were released from the melanoma biopsy by macerating the biopsy with the plunger of a 10ml syringe. The resulting melanoma cell suspension was purified on a Ficol-
10 Hypaque (Amersham Pharmacia, Uppsala, Sweden) gradient. Contaminating fibroblasts and leucocytes were removed by mixing with Dynal beads coated with monoclonal antibodies (Mab's) to human fibroblasts (Cat#, MAS516X, SeraLab) and to the leucocyte common antigen (CD45, Cat# 17-0804-3, Amrad Biotech, Victoria, Australia).

Subsequently, 1×10^6 cells were placed into wells of a 24-well tissue culture plate
15 and inoculated with approximately 1×10^5 plaque forming units of CAV21 prepared in Example 1. Following incubation at 37°C for 20 hours, cells were assessed for cell death by staining with propidium iodine and microscopic analysis.

Fig. 5 shows that both adherent and suspension primary melanoma cells were efficiently killed as a result of CAV21 infection during the 20 hour incubation period.

Example 5***5.1 Expression of ICAM-1 and DAF on Melanoma Cells Susceptible to CAV21******Infection***

To confirm melanoma cells are highly susceptible to infection and resultant killing
5 by CAV21, six additional human melanoma cell lines derived from primary human
melanomas were infected with CAV21 prepared in Example 1.

Fig. 6(A) indicates that all melanoma cell lines except one (ME 105) were killed as
a result of CAV21 infection during a 23 hour incubation period.

To confirm high level expression of ICAM-1 and DAF on the surface of malignant
10 melanoma cells, cells from each cell line were treated with the Mab IH4 and Mab
WEHI-CAM. The binding of the anti-DAF and anti-ICAM-1 Mab was detected by flow
cytometric analysis as described above. The fluorescence histograms shown in Fig. 6(B)
confirm high level expression of DAF and ICAM-1 on the surface of all melanoma cell
lines examined except the ME 105 cell line. The lack of DAF and ICAM-1 expression
15 rendered this cell line refractile to CAV21 infection.

Example 6***6.1 Selective Infection of Melanoma Cells Expressing ICAM-1***

To highlight the selective nature of CAV21 infection of ICAM-1 expressing
20 human melanoma cells, monolayers of melanoma cell line MM 200 were inoculated
with approximately 1×10^5 plaque forming units of CAV21, Coxsackievirus B3
(CVB3), Echovirus type 7 (E7) or Coxsackievirus B1 (CVB1) in wells of a 24-well
tissue culture plate for one hour at 37°C, respectively. The viral inoculate was

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subsequently removed and the cell monolayers then washed with phosphate buffered saline (PBS), and 1.0 ml of DMEM containing 1.0% foetal calf serum was added to each well and the cells incubated at 37°C for 48 hours. To quantitate cell survival, monolayers were incubated with a crystal violet/methanol solution, washed with distilled water and microscopically examined at 100 X.

Fig. 7 shows that following the 48 hour incubation period only CAV21 infected the MM 200 melanoma cells while the reverse occurred in the rhabdomyosarcoma cells (RD) where CVB1, CVB3 and E7 infection and killing is evident. RD cells express DAF but no ICAM-1. However, when ICAM-1 is expressed on the surface of RD cells they are highly susceptible to CAV21 induced infection and killing.

Example 7

7.1 Infection of Melanoma Biopsy With CAV21

Sections of solid human melanoma lymph node biopsies were placed in wells of a 24-well tissue culture plate and mock infected or challenged with approximately 1×10^5 plaque forming units of CAV21 or CVB3.

The results shown in Fig. 8 indicate that CAV21 infection resulted in severe tissue destruction around the perimeter of the melanoma biopsy treated with that virus while no detectable viral membrane destruction was observed in the mock and CVB3 infected biopsies.

Example 8

8.1. Lytic Infection of Human Melanoma Cells by CAV21 and CAV15

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To assay the oncolytic potential of CAV15 and CAV21 on human melanoma cell lines, Sk-Mel-28 and ME4405 cells were seeded into flat-bottom 96-well microtiter plates (Becton Dickinson) at 3×10^4 cells per well. Following incubation for 24 h at 37°C, culture medium was removed and replaced with fresh medium containing the appropriate viral serial dilution in a final volume of 100 µl. Stock viral preparations were serially diluted 10^{-1} through to 10^{-7} . Following viral inoculation, the plates were incubated at 37°C for 48 h and cell survival was detected by crystal violet staining as described above.

All three cell lines RD-ICAM-1, Sk-Mel-28 and ME4405 were found to be permissive to lytic infection by both CAV21 and CAV15. Following an incubation period of 48 h, the no virus control showed no signs of viral induced CPE while extensive cell lysis was observed across all cell cultures at a dilution of 10^{-1} and 10^{-2} . At higher viral dilutions Sk-Mel-28 cells were shown to be more permissive to viral lysis compared to ME4405 and RD-ICAM-1 cell lines.

The overall oncolytic potential of CAV21 and CAV15 was higher in the melanoma cell lines, compared to the control RD-ICAM-1 cells. While all cell types express similar levels of ICAM-1, DAF expression in RD-ICAM-1 cells is significantly lower than on melanoma cells (see Fig. 2) accounting for lower viral attachment via DAF to RD-ICAM-1 cells. DAF has previously been shown to be a low affinity sequestration molecule for many Coxsackieviruses, assisting the capture of virus particles and hence infectivity of the cells (Lea SM, et al; 1998). The presence of higher levels of DAF expression on the melanoma cell lines compared to the RD-ICAM-1 cells increases the

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probability of viral access to ICAM-1 receptors, thus leading to an increased level of infection and cell lysis.

8.2. Lytic Infection of Human Prostate Cancer Cells by Coxsackievirus

Cells from the human prostate cancer cell line CP3 (which expresses ICAM-1) were seeded into a flat-bottom 96-well microtitre plate (Becton Dickinson) at 3×10^4 cells per well and treated with serial dilutions of CAV13, CAV15, CAV21 and the Coxsackievirus B-group virus CVB3 following incubation of the cells, as described in Example 8.1 above. PC3 cells are available from the American Type Culture Collection (ATCC) Manassas, Virginia, USA under Accession No. CRL-1435.

As shown in Fig. 9, the PC3 cells were highly permissive to lytic infection by CAV15. Extensive lytic infection was also observed for both CAV13 and CAV21.

8.3. Selective Replication of CAV21 and CAV15 in the Human Melanoma Cell Lines Sk-Mel-28 and ME4405

The selectivity of CAV21 and CAV15 for the melanoma cell lines Sk-Mel-28 and ME4405 was studied using an *in vitro* specificity assay.

Sterile cell culture inserts were used to divide the wells of a standard six well plate tissue culture plate. Inside the cell culture insert, either Sk-Mel-28 cells or ME4405 cells were grown, with MRC5 or RD cells grown around the cell culture insert. Once the cells had adhered, the cell culture inserts were removed from each of the well allowing the cell culture media to evenly cover the co-culture. When the perimeters of both cell populations had fused, the co-cultures were washed twice with PBS and then inoculated with 500 μ l of either PBS or stock virus (10^5 TCID₅₀) for 1 h at 37°C. Following incubation at 37°C, fresh DMEM containing 1% FCS was added to each of

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the wells and the plates incubated for 48 h at 37°C in a 5% CO₂ atmosphere. Cell monolayers were monitored by light microscopy for signs of virus-induced CPE, prior to each well being stained with 3 ml of crystal violet solution for the detection of cell survival from viral induced lytic infection. The capacity of CAV21 and CAV15 viruses to specifically lytically destroy melanoma cells without infecting non-melanoma surrounding cells is illustrated in Figure 10.

As can be seen, the inner cultures of melanoma cells in each well treated with CAV21 or CAV15 were totally destroyed by the viruses, but were unaffected by CVB3 virus which does not employ ICAM-1 as a receptor for cell entry. CVB3 which employs the Coxsackie-and adenovirus receptor (CAR) for cell entry (10). MRC5 cells appeared to be refractory to lytic infection by both CAV21 and CAV15. These cells are derived from a human lung fibroblast culture and only express low levels of ICAM-1 (unpublished data). The present data shows that rapid and effective lytic infection of target cells facilitated high level ICAM-1 and DAF expression. RD cells, which do not express ICAM-1, were not destroyed by either CAV21 or CAV15 infection. Furthermore, the results show little if any spread of CAV21 and CAV15 to receptor negative cells that are in direct contact with virally infected receptor-bearing cells.

Example 9

The lytic infection of preformed melanoma tumours *in vivo* was evaluated by a series of animal challenge experiments using NOD-SCID mice.

9.1. Development of Melanoma Xenografts in NOD-SCID Mice

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All animal work was performed under guidelines approved by The University Of Newcastle Animal Care and Ethics Committee. NOD-SCID mice were housed in pathogen-free quarters in the animal handling facility located at the David Maddison Building, Level 5, Newcastle, NSW, Australia.

5 Sk-Mel-28 and ME4405 cells were grown in DMEM containing 10% FCS. The cells were harvested and washed twice with DMEM, and resuspended in sterile PBS. The cell concentration of the suspension was determined with a haemocytometer and cell viability was assessed by trypan blue staining. Only cell preparations with >95% viability were used for xenotransplantation. Prior to xenotransplantation, animals were
10 anaesthetised with intraperitoneal (i.p) injections of Rompun/Ketamine (50 mg/kg). For the monitoring of animals and measurement of tumour growth, animals were anaesthetised with 3% isofluorane.

The tumour cells were xenografted into the flank of anaesthetised 4-6 week old female NOD-SCID mice. Xenograft tumour growth was observed daily and measured
15 with callipers at various intervals with all measurements recorded in millimetres over the course of 5 weeks. Estimates of tumour volumes were calculated using known methods (Davies CD, et al; 1997).

9.2. Subcutaneous Viral Delivery

In a preliminary experiment employing fifteen NOD-SCID mice, the local
20 subcutaneous delivery of virus through *ex vivo* infected cells was assessed for inhibition of tumour growth. The mice in the control group (n=5) were injected subcutaneously with Sk-Mel-28 cells (1×10^7) cells at individual sites in both the upper and lower flank. The CAV21 group (n=5) received an injection of 1×10^7 Sk-Mel-28 cells in the upper

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flank and a second injection of Sk-Mel-28 (1×10^7) cells that had been pre-incubated with 10^4 TCID₅₀ of CAV21 at room temperature for 1 hour *ex vivo*. The CAV15 group (n=5), was treated the same as the CAV21 group except that the second injection in the lower flank contained Sk-Mel-28 (1×10^7) cells that had been incubated with 10^4 TCID₅₀ of CAV15. Four weeks post-injection, a representative of the control group was sacrificed and shown to bear two individual tumour masses corresponding to the two injection sites of the Sk-Mel-28 (1×10^7) cells. In contrast a representative of the CAV21 group beared no detectable tumour formation in either the uninfected cell or virally infected cell sites of injection (Figure 11). Upon autopsy examination, all remaining members of the control group were shown to possess two distinct melanoma xenograft tumour growths, while remaining members of the CAV21 group (17 weeks post injection) exhibited no detectable tumour growth in either site of injection. Mice in the CAV15 group exhibited no tumour formation at 4 weeks post- injection.

9.3. Intratumoural Viral Delivery

Twenty NOD-SCID mice were injected with Sk-Mel 28 cells (1×10^7) in the upper flank. When the tumour volume reached ~ 50 - 100 mm^3 the animals were randomly divided into groups of five and housed in separate cages. Groups of mice were injected intratumourally with 100 μl of active CAV21 or CAV15 containing $10^{3.2}$ or $10^{4.2}$ TCID₅₀ doses, respectively. The remaining animals received 100 μl of PBS injected directly into the xenografts. The different treatment groups were housed in individually vented cages maintained under negative pressure, ensuring that virus and other pathogens were contained within the individual cages.

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A dose of $10^{3.2}$ or $10^{4.2}$ TCID₅₀ of either CAV21 or CAV15 respectively, was sufficient to produce significant tumour reduction in animals bearing preformed Sk-Mel-28 tumours at 14 days post-injection. The trend of reduction of tumour burden continued for the next 14-21 days. No detectable tumours were observed at 30-35 days post-injection (see Figures 12 and 13). The difference observed between the CAV21 treated group and the PBS treated control group was statistically significant ($P=0.0023$, t test). Animals bearing Sk-Mel-28 tumours and injected with CAV21 showed no clinical signs of CAV21 illness. The capacity of CAV15 to drastically reduce melanoma tumour burden is shown in Figure 14. At 35 days post-injection, the melanoma xenograft treated with PBS was approximately 2037 mm³ while the CAV15 treated tumour was approximately 2 mm³ in volume ($P=0.0053$, t test). The CAV15 treated tumour shown comprises mostly residual connective tissue.

9.4. Intratumoral Delivery of CAV21 to ME4405 Xenograft

The intratumoural delivery of CAV21 to a different melanoma (ME4405) xenograft was undertaken to further confirm the anti-tumour therapy potential of this virus. Fifteen NOD-SCID mice were injected with ME4405 cells (5×10^6) subcutaneously in a single site on the flank. When tumour volumes had reached approximately 500 mm³, the animals were randomly divided into groups of five and housed in separate cages. Five animals were injected intratumourally with 100 µl of active CAV21 containing $10^{3.2}$ TCID₅₀ doses, while five mice received 100 µl of PBS injected directly into the xenografts and the remaining five mice were left untreated. As shown in Fig. 15, intratumoural administration of CAV21 was able to markedly reduce tumour development of ME4405 cells within 25 days post-injection even though the initial pre-

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injection tumour volume was 5-fold greater than those utilised above. The ME4405 xenografts were observed to be more aggressive than the Sk-Mel-28 tumours as assessed by significantly faster growth rates of tumours in the control groups.

The ME4405 cell line generated highly vascular aggressive tumours compared to Sk-Mel-28 tumours which grew at a slower rate and were not as vascular as the ME4405 tumours.

In contrast to mice bearing Sk-Mel-28 xenografts, when CAV21 was injected into animals with ME4405 tumours, some signs of illness were observed, the most notable being a transient weakness in both the fore and hind limbs. No positional abnormalities were observed.

9.5. Discussion of Results

This study demonstrates that CAV13, CAV15 and CAV21 have the capacity to lytically destroy malignant cell lines.

Specifically, the *in vitro* analysis of CAV21 and CAV15 infection of melanoma cells shows that these two viruses are able to selectively infect Sk-Mel-28 and ME4405 cell lines as a result of the expression of ICAM-1 and DAF while each of the Cocksackieviruses mentioned above were able to infect and cause the death of cells of the prostate cancer line PC3. Moreover, the intratumoural injection of CAV21 and CAV15 into xenografts of human melanoma cell lines grown in the flanks of NOD-SCID mice show that CAV21 and CAV15 possess therapeutic applications against malignant melanoma. The direct injection of either of the two viruses into pre-formed melanoma tumours suppressed tumour growth and led to significant tumour regression and in some cases complete tumour destruction compared to control animals. Furthermore, the

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delivery of cells infected by virus *ex vivo* yielded total inhibition of tumour growth and demonstrates that *ex vivo* CAV21 infected melanoma cells are capable of delivering sufficient virus to inhibit local tumour growth. In addition, injection of infected cells subcutaneously in a distant region to the initial tumour challenge shows that the virus
5 can travel systemically.

The pathogenesis of CAV21 and CAV15 infections are mainly asymptomatic or manifest by no more than minor malaise. The Coe strain of CAV21 has recently been approved for live administration by the Food and Drug Administration (FDA) of the United States of America for the clinical assessment of specific anti-viral agents against
10 CAV21 (90). The recent development of specific antiviral agents against CAV21 and CAV15 provides the added safety precaution of drug intervention to control viral infection.

Although the present invention has been described hereinbefore with reference to a number of preferred embodiments, the skilled addressee will understand that numerous
15 modifications and variations are possible without departing from the scope of the invention.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A method of treating abnormal cells in a mammal comprising administering to the mammal an effective amount of a virus capable of infecting the abnormal cells whereby
5 death of the cells is caused and which recognises at least one of a cell adhesion molecule of the immunoglobulin (Ig) superfamily and a complement regulatory protein for infectivity of the abnormal cells.
2. A method according to claim 1 wherein the cell adhesion molecule is ICAM-1.
3. A method according to claim 1 or 2 wherein the virus recognises both the cell
10 adhesion molecule and the complement regulatory protein for infectivity of the abnormal cells.
4. A method according to claim 1 wherein the virus recognises the complement regulatory protein for infectivity of the abnormal cells.
5. A method according to claim 3 or 4 wherein the complement regulatory protein is
15 DAF.
6. A method according to any one of claims 1 to 3 wherein expression of the cell adhesion molecule is upregulated on the abnormal cells relative to normal said cells expressing their normal phenotype.
7. A method according to any one of claims 1 to 3 wherein expression of the cell
20 adhesion molecule is upregulated on the abnormal cells relative to cells of surrounding tissue in which the abnormal cells are found.
8. A method according to any one of claims 1 to 7 wherein the virus is an animal RNA virus.

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9. A method according to any one of claims 1 to 8 wherein the virus is an Enterovirus.
10. A method according to claim 9 wherein the virus is a Coxsackievirus.
11. A method according to claim 10 wherein the virus is selected from the group
- 5 consisting of CAV-13, CAV-15, CAV-18 and CAV-21.
12. A method according to any one of claims 1 to 11 wherein the virus is a recombinant virus.
13. A method according to any one of claims 1 to 12 wherein the abnormal cells are cells of a malignancy selected from the group consisting of a malignancy of the skin,
- 10 prostate cancer, stomach cancer, breast cancer and colon cancer.
14. A method according to any one of claims 1 to 13 wherein the virus is administered intravenously, intratumorally, intraperitoneally, intramuscularly, or by topical application.
15. A method according to any one of claims 1 to 14 wherein the virus is administered
- 15 by injection.
16. A method according to any one of claims 1 to 15 wherein the virus is administered to the patient in a dosage greater than about 1×10^2 plaque forming units per ml of inoculant.
17. A method according to claim 16 wherein the virus is administered to the patient in
- 20 a dosage of between about 1×10^2 to 1×10^{10} plaque forming units per ml of the inoculant.
18. A method of treating melanoma in a patient comprising administering to the patient an effective amount of a virus capable of infecting melanoma cells whereby death

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of the cells is caused and wherein the virus recognises at least one of a cell adhesion molecule and a complement regulatory protein for infectivity of the melanoma cells.

19. A method according to claim 18 wherein the cell adhesion molecule is a member of the immunoglobulin (Ig) superfamily.

5 20. A method according to claim 18 or 19 wherein the cell adhesion molecule is ICAM-1.

21. A method according to any one of claims 18 to 20 wherein the virus recognises the cell adhesion molecule for infectivity of the melanoma cells.

22. A method according to any one of claims 18 to 21 wherein the virus recognises
10 both the cell adhesion molecule and the complement regulatory protein for infectivity of the melanoma cells.

23. A method according to claim 18 wherein the virus recognises the complement regulatory protein for infectivity of the melanoma cells.

24. A method according to claim 22 or 23 wherein the complement regulatory protein
15 is DAF.

25. A method according to any one of claims 18 to 24 wherein the virus is an animal RNA virus.

26. A method according to any one of claims 18 to 25 wherein the virus is a member of the *Picornaviridae* family.

20 27. A method according to any one of claims 18 to 26 wherein the virus is a Coxsackivirus.

28. A method according to claim 27 wherein the virus is selected from the group consisting of CAV-13, CAV-15, CAV-18 and CAV-21.

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29. A method according to any one of claims 18 to 28 wherein the virus is administered intravenously, intratumorally, intraperitoneally, intramuscularly, or by topical application.
30. A method according to claim 29 wherein the virus is administered by injection.
- 5 31. A method according to any one of claims 18 to 30 wherein the virus is administered to the patient in a dosage greater than about 1×10^2 plaque forming units per ml of inoculant.
32. A method according to claim 31 wherein the virus is administered to the patient in a dosage of between about 1×10^2 to 1×10^{10} plaque forming units per ml of the
- 10 inoculant.
33. A method of screening abnormal cells for determining whether the cells are susceptible to viral induced cell death, comprising the steps of:
- (a) providing the abnormal cells;
 - (b) adding to the cells an effective amount of a virus which recognises at least one
 - 15 of a cell adhesion molecule of the immunoglobulin (Ig) superfamily and a complement regulatory protein for infectivity of the abnormal cells;
 - (c) incubating the abnormal cells in the presence of the virus for a period of time; and
 - (d) determining whether the virus has infected and caused death of at least some
 - 20 of the abnormal cells.
34. A method according to claim 33 wherein the cell adhesion molecule is ICAM-1.

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35. A method according to claim 33 or 34 wherein the virus recognises both the cell adhesion molecule and the complement regulatory protein for infectivity of the abnormal cells.
36. A method according to claim 33 wherein the virus recognises the complement regulatory protein for infectivity of the abnormal cells.
37. A method according to claim 35 or 36 wherein the complement regulatory protein is DAF.
38. A method according to any one of claims 33 to 37 wherein expression of the cell adhesion molecule is upregulated on the abnormal cells relative to normal said cells expressing their normal phenotype.
39. A method according to any one of claims 33 to 37 wherein expression of the cell adhesion molecule is upregulated on the abnormal cells relative to cells of surrounding tissue in which the abnormal cells are found.
40. A method according to any one of claims 33 to 39 wherein the virus is an animal RNA virus.
41. A method according to any one of claims 33 to 40 wherein the virus is a member of the *Picornaviridae* family.
42. A method according to any one of claims 33 to 41 wherein the virus is an Enterovirus.
43. A method according to claim 42 wherein the virus is a Cocksackievirus.
44. A method according to any one of claims 33 to 43 wherein the abnormal cells are cells of a malignancy selected from the group consisting of a malignancy of the skin, prostate cancer, stomach cancer, breast cancer and colon cancer.

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45. A method of screening melanoma cells for determining whether the cells are susceptible to viral induced cell death, comprising the steps of:

(a) providing the melanoma cells;

(b) adding to the melanoma cells an effective amount of a virus which recognises

5 at least one of a cell adhesion molecule and a complement regulatory protein for infectivity of the melanoma cells;

(c) incubating the melanoma cells in the presence of the virus for a period of time;

and

(d) determining whether the virus has infected and caused death of at least some

10 of the melanoma cells.

46. A method according to claim 45 wherein the cell adhesion molecule is a member of the immunoglobulin (Ig) super family.

47. A method according to claim 46 wherein the cell adhesion molecule is ICAM-1.

48. A method according to any one of claims 45 to 47 wherein the virus recognises the
15 cell adhesion molecule for infectivity of the melanoma cells.

49. A method according to any one of claims 45 to 48 wherein the virus recognises both the cell adhesion molecule and the complement regulatory protein for infectivity of the melanoma cells.

50. A method according to claim 45 wherein the virus recognises the complement
20 regulatory protein for infectivity of the melanoma cells.

51. A method according to any one of claims 45 to 50 wherein the complement regulatory protein is DAF.

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52. A method according to any one of claims 45 to 51 wherein the virus is a member of the *Picornaviridae* family.

53. A method according to claim 52 wherein the virus is an Enterovirus.

54. A method according to claim 53 wherein the virus is a Cocksackievirus.

5 55. A method of screening a virus for ability to infect and cause death of abnormal cells, comprising the steps of:

(a) selecting a virus which recognises at least one of a cell adhesion molecule of the (Ig) superfamily and a complement regulatory protein for infectivity of the abnormal cells;

10 (b) incubating the selected said virus with a sample of the abnormal cells for a period of time; and

(c) determining whether the selected said virus causes death of at least some of the abnormal cells.

56. A method according to claim 55 further comprising comparing the ability of the
15 virus to infect and cause the death of the abnormal cells with that of a different said virus subjected to steps (b) and (c) utilising another sample of the cells.

57. A method according to claim 55 or 56 wherein the cell adhesion molecule is ICAM-1.

58. A method according to any one of claims 55 to 57 wherein the virus recognises the
20 cell adhesion molecule for infectivity of the abnormal cells.

59. A method according to any one of claims 55 to 58 wherein the virus recognises both the cell adhesion molecule and the complement regulatory protein for infectivity of the abnormal cells.

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60. A method according to claim 55 wherein the virus recognises the complement regulatory protein for infectivity of the abnormal cells.
61. A method according to claim 59 or 60 wherein the complement regulatory protein is DAF.
- 5 62. A method according to any one of claims 55 to 61 wherein expression of the cell adhesion molecule is upregulated on the abnormal cells relative to normal said cells expressing their normal phenotype.
63. A method according to any one of claims 55 to 62 wherein the virus is an animal RNA virus.
- 10 64. A method according to any one of claims 55 to 63 wherein the virus is a member of the *Picornaviridae* family.
65. A method according to claim 64 wherein the virus is a Cocksackievirus.
66. A method according to any one of claims 55 to 59 wherein the abnormal cells are cells of a malignancy selected from the group consisting of a malignancy of the skin, prostrate cancer, stomach cancer, breast cancer and colon cancer.
- 15 67. A method of screening a virus for ability to infect and cause death of melanoma cells, comprising the steps of:
- (a) selecting a virus which recognises at least one of a cell adhesion molecule and a complement regulatory protein for infectivity of the melanoma cells;
- 20 (b) incubating the selected said virus with a sample of the melanoma cells for a period of time; and
- (c) determining whether the selected said virus causes death of at least some of the melanoma cells.

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68. A method according to claim 67 further comprising comparing the ability of the virus to infect and cause the death of the melanoma cells with that of a different said virus subjected to steps (b) and (c) utilising another sample of the cells.
69. A method according to claim 67 or 68 wherein the cell adhesion molecule is a member of the immunoglobulin (Ig) superfamily.
70. A method according to any one of claims 67 to 69 wherein the cell adhesion molecule is ICAM-1.
71. A method according to any one of claims 67 to 69 wherein the virus recognises both the cell adhesion molecule and the complement regulatory protein for infectivity of the melanoma cells.
72. A method according to claim 67 or 68 wherein the virus recognises the complement regulatory protein for infectivity of the melanoma cells.
73. A method according to claim 71 or 72 wherein the complement regulatory protein is DAF.
74. A method according to any one of claims 67 to 73 wherein the virus is an animal RNA virus.
75. A method according to any one of claims 67 to 74 wherein the virus is a member of the *Picornaviridae* family.
76. A method according to claim 75 wherein the virus is an Enterovirus.
77. A method according to claim 76 wherein the virus is a Cocksackievirus.
78. A pharmaceutical composition comprising a pharmaceutically acceptable carrier together with a virus capable of infecting abnormal cells whereby death of the cells is caused and which recognises at least one of a cell adhesion molecule of the

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immunoglobulin (Ig) superfamily and a complement regulatory protein for infectivity of the abnormal cells.

79. A pharmaceutical composition according to claim 78 wherein the cell adhesion molecule is ICAM-1.

5 80. A pharmaceutical composition according to claim 78 or 79 wherein the virus recognises the cell adhesion molecule for infectivity of the abnormal cells.

81. A pharmaceutical composition according to any one of claims 78 to 80 wherein the virus recognises both the cell adhesion molecule and the complement regulatory protein for infectivity of the abnormal cells.

10 82. A pharmaceutical composition according to claim 78 wherein the virus recognises the complement regulatory protein for infectivity of the abnormal cells.

83. A pharmaceutical composition according to claim 82 wherein the complement regulatory protein is DAF.

84. A pharmaceutical composition according to any one of claims 78 to 81 wherein
15 expression of the cell adhesion molecule is upregulated on the abnormal cells relative to normal said cells expressing their normal phenotype.

85. A pharmaceutical composition according to any one of claims 78 to 84 wherein the virus is an animal RNA virus.

86. A pharmaceutical composition according to any one of claims 78 to 85 wherein the
20 virus is a member of the *Picornaviridae* family.

87. A pharmaceutical composition according to claim 86 wherein the virus is an Enterovirus.

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88. A pharmaceutical composition according to claim 87 wherein the virus is a Coxsackievirus.
89. A pharmaceutical composition according to any one of claims 78 to 88 wherein the abnormal cells are malignant cells of a malignancy selected from the group consisting of
5 a malignancy of the skin, prostate cancer, stomach cancer, breast cancer and colon cancer.
90. A pharmaceutical composition comprising a pharmaceutically acceptable carrier together with a virus capable of infecting melanoma cells whereby death of the cells is caused and which recognises at least one of a cell adhesion molecule and a complement
10 regulatory protein for infectivity of the melanoma cells.
91. A pharmaceutical composition according to claim 90 wherein the cell adhesion molecule is a member of the immunoglobulin (Ig) superfamily.
92. A pharmaceutical composition according to claim 90 or 91 wherein the cell adhesion molecule is ICAM-1.
- 15 93. A pharmaceutical composition according to any one of claims 90 to 92 wherein the virus recognises the cell adhesion molecule for infectivity of the melanoma cells.
94. A pharmaceutical composition according to any one of claims 90 to 93 wherein the virus recognises both the cell adhesion molecule and the complement regulatory protein for infectivity of the melanoma cells.
- 20 95. A pharmaceutical composition according to claim 90 wherein the virus recognises the complement regulatory protein for infectivity of the melanoma cells.
96. A pharmaceutical composition according to claim 95 wherein the complement regulatory protein is DAF.

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97. A pharmaceutical composition according to any one of claims 90 to 96 wherein the virus is an animal RNA virus.
98. A pharmaceutical composition according to any one of claims 90 to 97 wherein the virus is a member of the *Picornaviridae* family.
- 5 99. A pharmaceutical composition according to claim 98 wherein the virus is an Enterovirus.
100. A pharmaceutical composition according to claim 99 wherein the virus is a Coxsackievirus.
101. A pharmaceutical composition according to claim 100 wherein the Coxsackievirus
- 10 is selected from the group consisting of CAV-13, CAV-15, CAV-18 and CAV-21.
102. A delivery means for being held against the skin of a patient and which is impregnated with a pharmaceutical composition as defined in any one of claims 78 to 101 for contact with the skin.
103. A delivery means according to claim 102 comprising a patch, pad, wad or bandage.
- 15 104. A delivery means according to claim 102 or 103 adapted for sticking to the skin of the patient to thereby hold the pharmaceutical composition in contact with the skin.
105. Use of a virus capable of infecting abnormal cells whereby death of the cells is caused, in the manufacture of a medicament for treating the abnormal cells and wherein the virus recognises at least one of a cell adhesion molecule of the immunoglobulin (Ig)
- 20 superfamily and a complement regulatory protein for infectivity of the abnormal cells.
106. Use according to claim 105 wherein the virus recognises ICAM-1 for infectivity of the abnormal cells.

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107. Use according to claim 105 or 106 wherein the virus recognises both the cell adhesion molecule and the complement regulatory protein for infectivity of the abnormal cells.

108. Use according to claim 105 wherein the virus recognises the complement
5 regulatory protein for infectivity of the abnormal cells.

109. Use according to claim 107 or 108 wherein the complement regulatory protein is DAF.

110. Use according to any one of claims 105 to 107 wherein expression of the cell adhesion molecule is upregulated on the abnormal cells relative to non-malignant said
10 cells expressing their normal phenotype.

111. Use according to any one of claims 105 to 107 wherein expression of the cell adhesion molecule is upregulated on the abnormal cells relative to cells of surrounding tissue in which the abnormal cells are found.

112. Use according to any one of claims 105 to 111 wherein the virus is an animal RNA
15 virus.

113. Use according to any one of claims 105 to 112 wherein the virus is a member of the *Picornaviridae* family.

114. Use according to claim 113 wherein the virus is an Enterovirus.

115. Use according to claim 114 wherein the virus is a Coxsackievirus.

20 116. Use according to claim 115 wherein the virus is selected from the group consisting of CAV-13, CAV-15, CAV-18 and CAV-21.

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117. Use according to any one of claims 105 to 115 wherein the abnormal cells are cells of a malignancy selected from the group consisting of a malignancy of the skin, prostate cancer, stomach cancer, breast cancer and colon cancer.

118. Use of a virus capable of infecting melanoma cells whereby death of the cells is caused, in the manufacture of a medicament for treating melanoma and wherein the virus recognises at least one of a cell adhesion molecule and a complement regulatory protein for infectivity of the melanoma cells.

119. Use according to claim 118 wherein the cell adhesion molecule is a member of the immunoglobulin (Ig) superfamily.

120. Use according to claim 118 or 119 wherein the cell adhesion molecule is ICAM-1.

121. Use according to any one of claims 118 to 120 wherein the virus recognises the cell adhesion molecule for infectivity of the melanoma cell.

122. Use according to any one of claims 118 to 121 wherein the virus recognises both the cell adhesion molecule and the complement regulatory protein for infectivity of the melanoma cells.

123. Use according to claim 118 wherein the virus recognises the complement regulatory protein for infectivity of the melanoma cells.

124. Use according to claim 122 or 123 wherein the complement regulatory protein is DAF.

125. Use according to any one of claims 118 to 124 wherein the virus is an animal RNA virus.

126. Use according to any one of claims 118 to 125 wherein the virus is a member of the *Picornaviridae* family.

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127. Use according to claim 125 or 126 wherein the virus is an Enterovirus.

128. Use according to claim 127 wherein the virus is a Coxsackievirus.

129. Use according to claim 128 wherein the virus is selected from the group consisting of CAV-13, CAV-15, CAV-18 and CAV-21.

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Figure 1

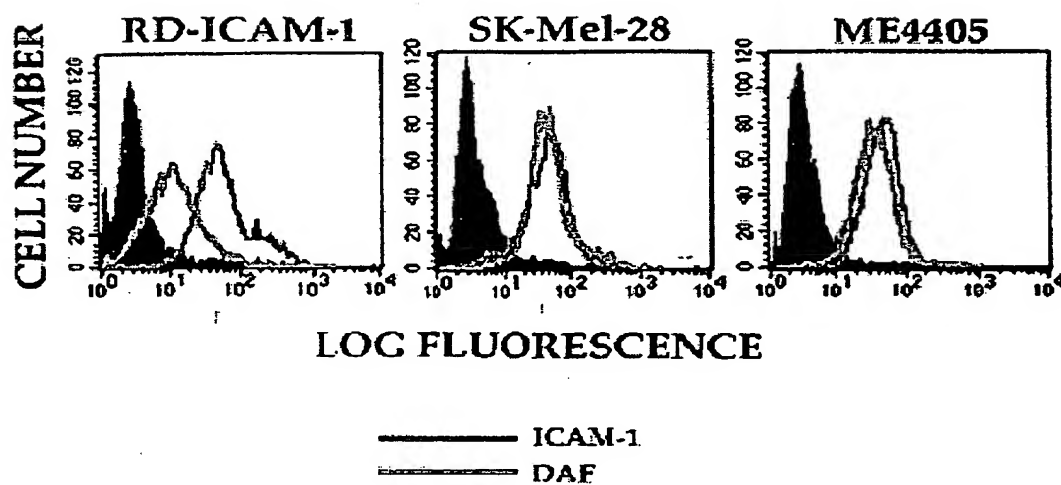


Figure 2

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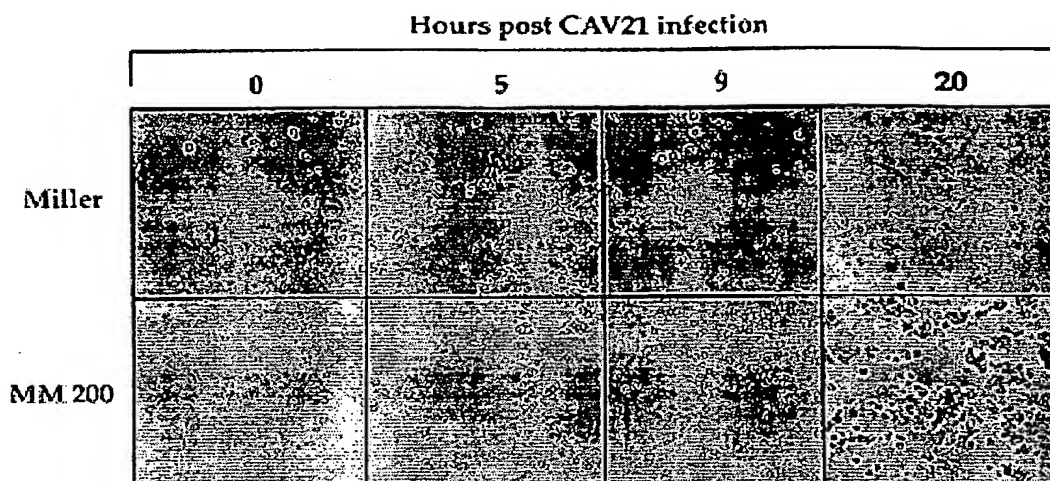


Figure 3

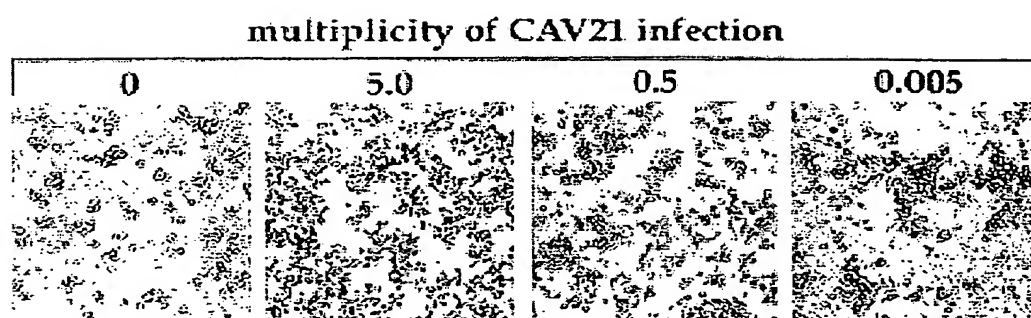


Figure 4

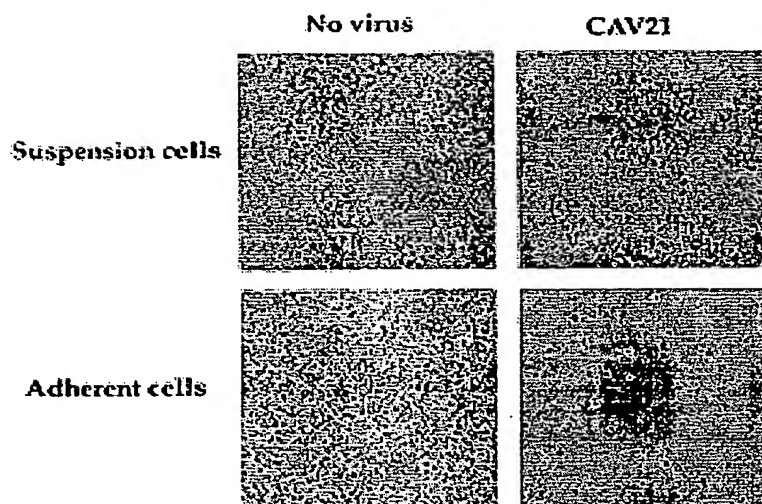


Figure 5

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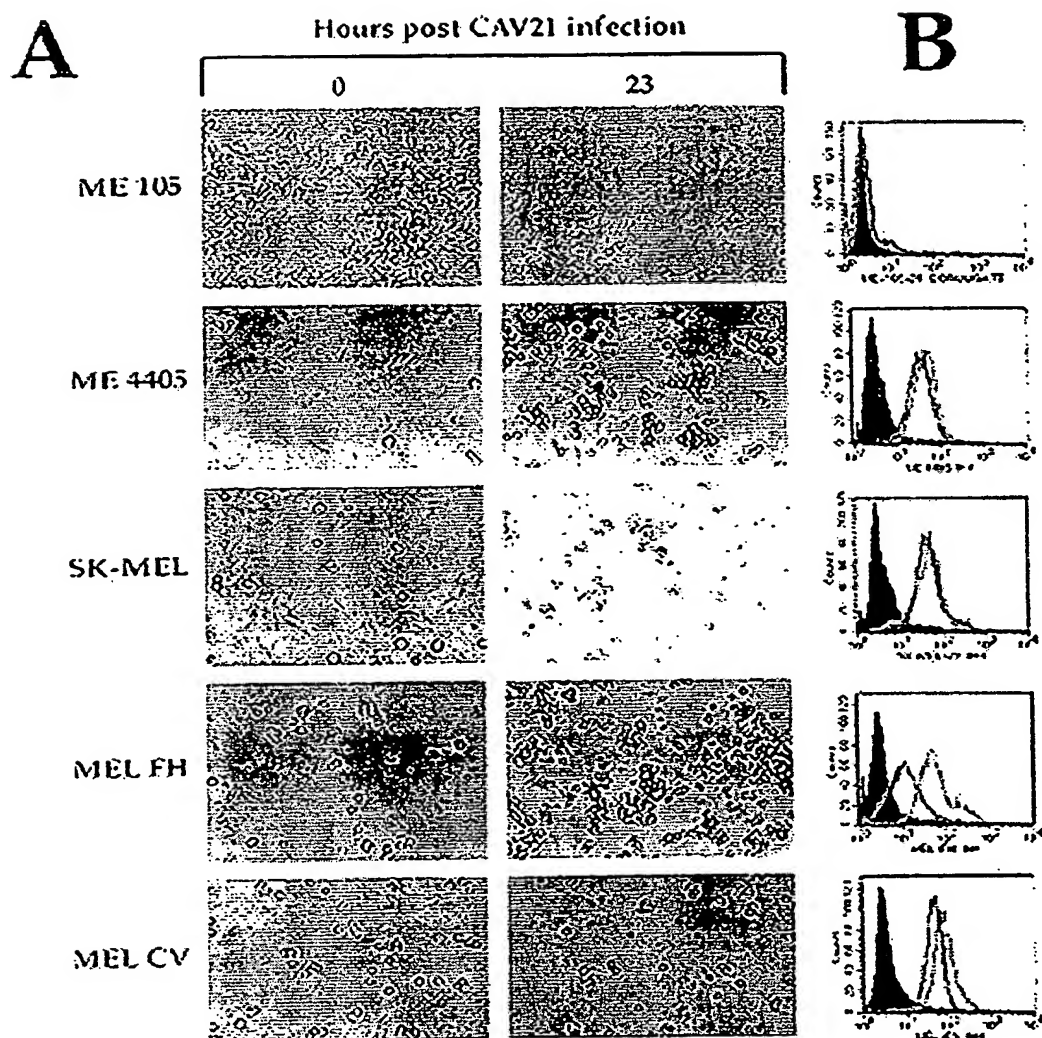


Figure 6

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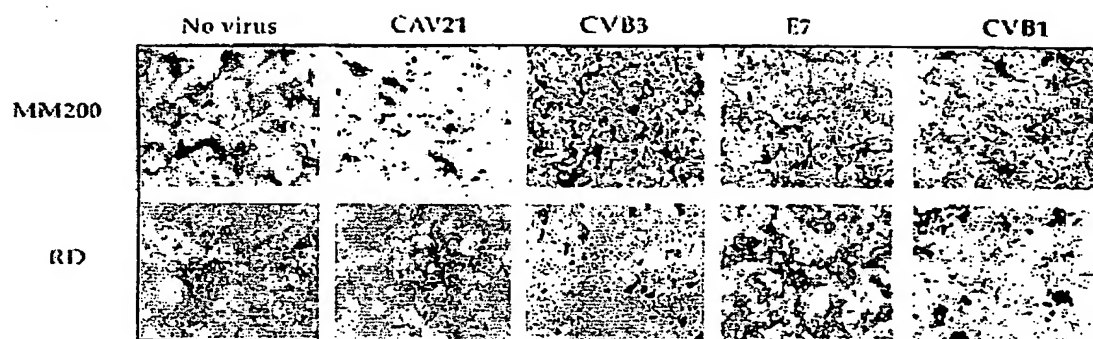


Figure 7

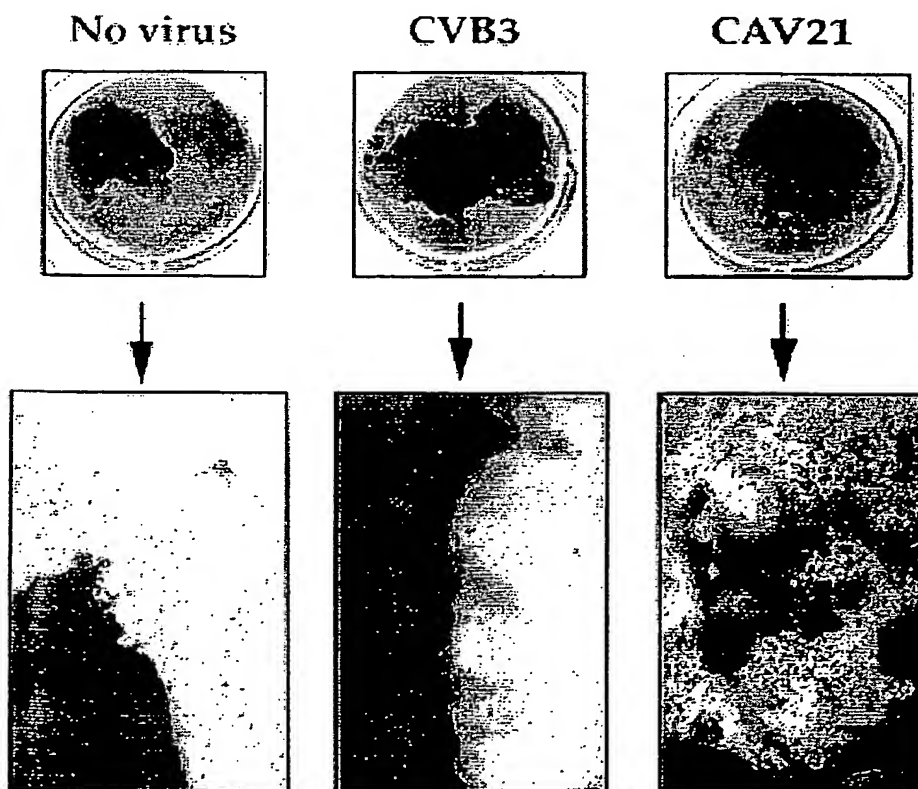
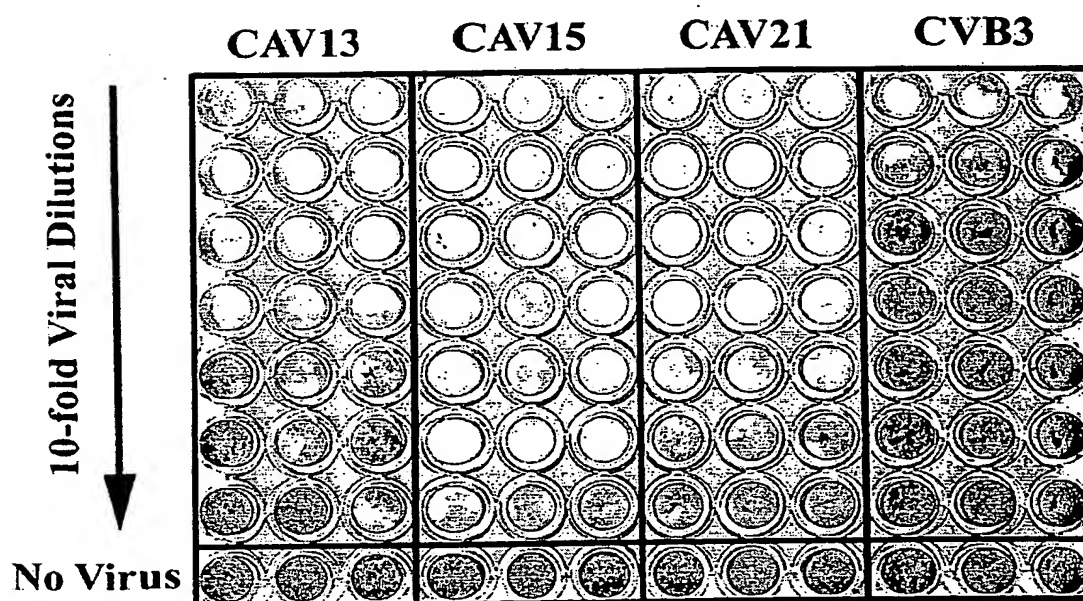


Figure 8

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Oncolytic Potential of Viruses on PC3 cells**Figure 9**

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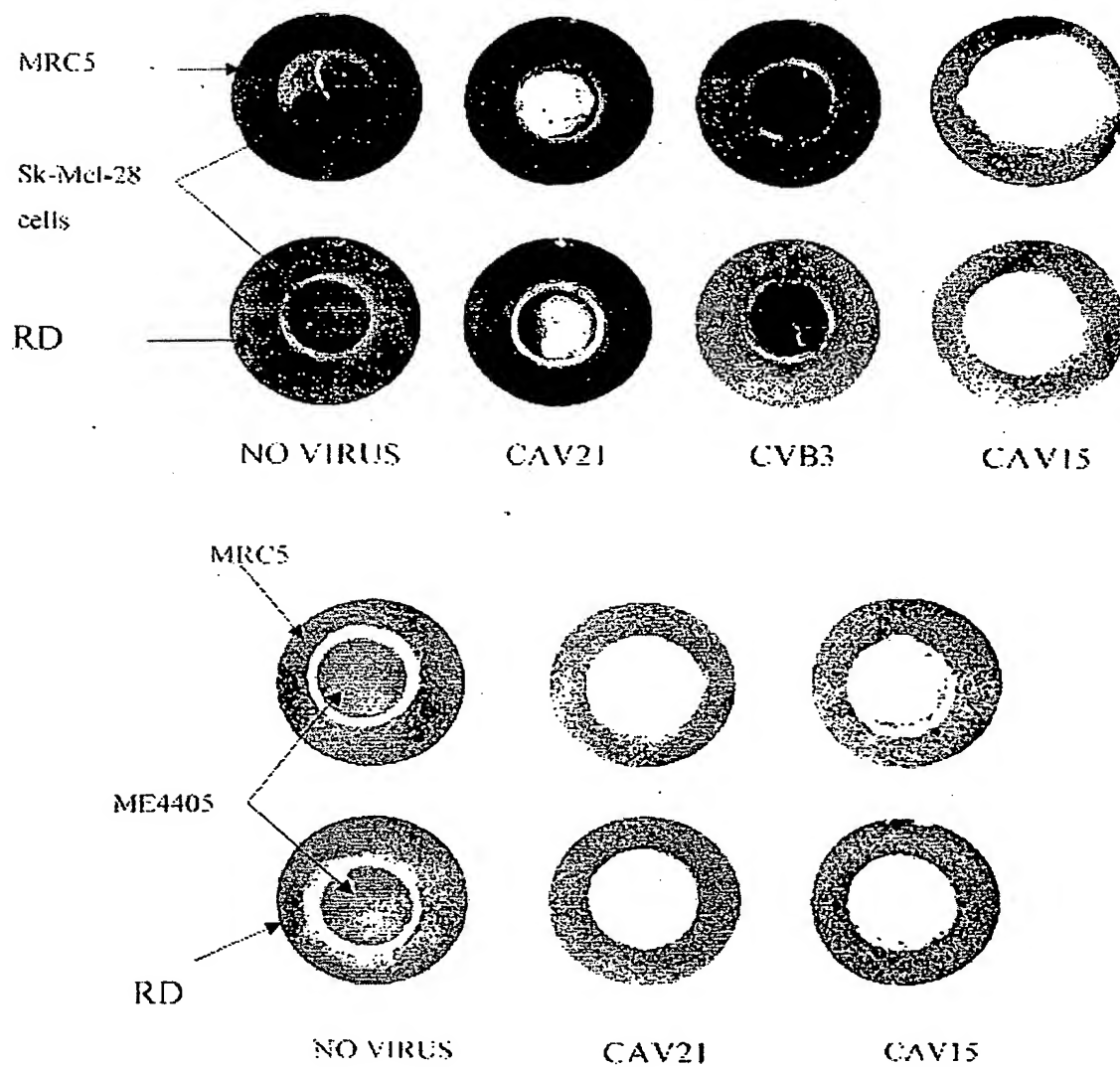


Figure 10

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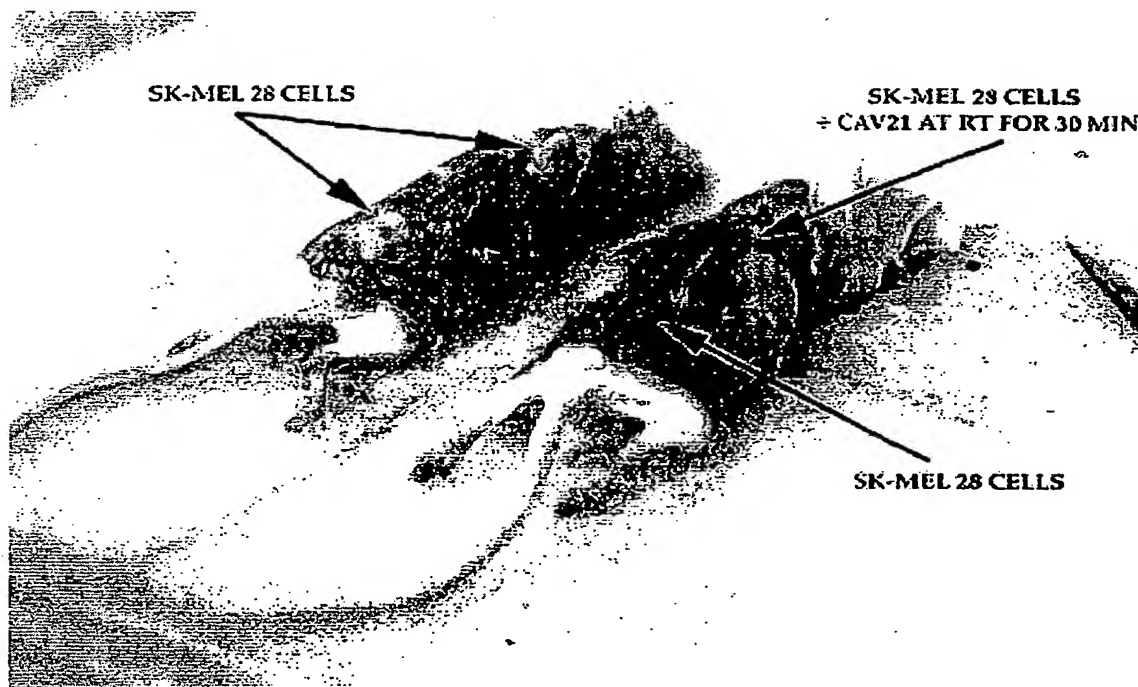


Figure 11

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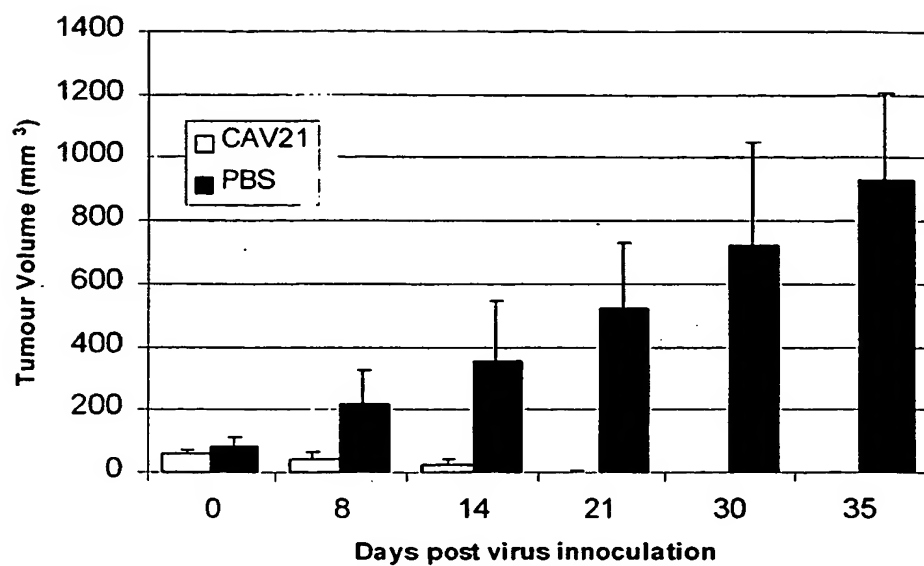


Figure 12

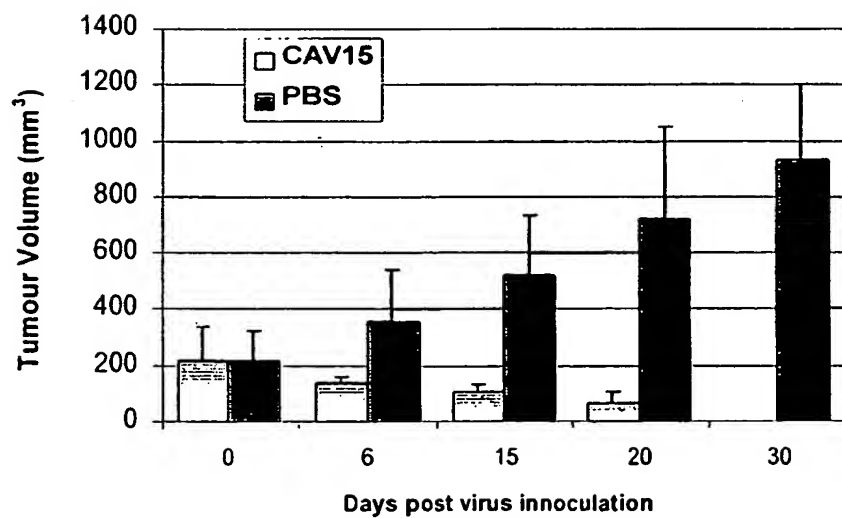


Figure 13

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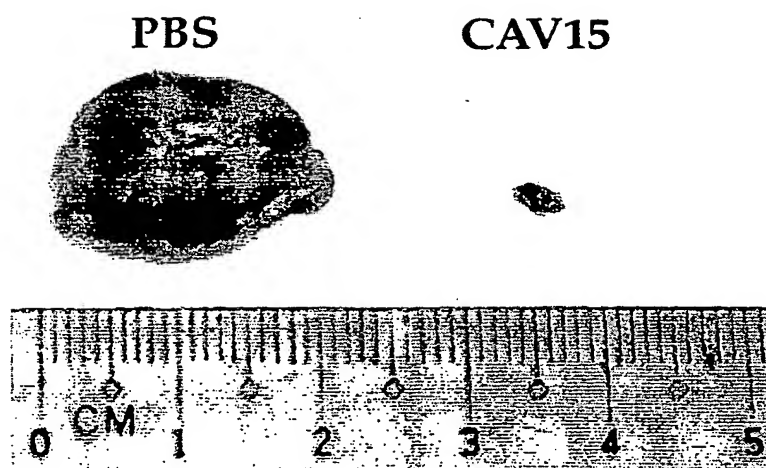


Figure 14

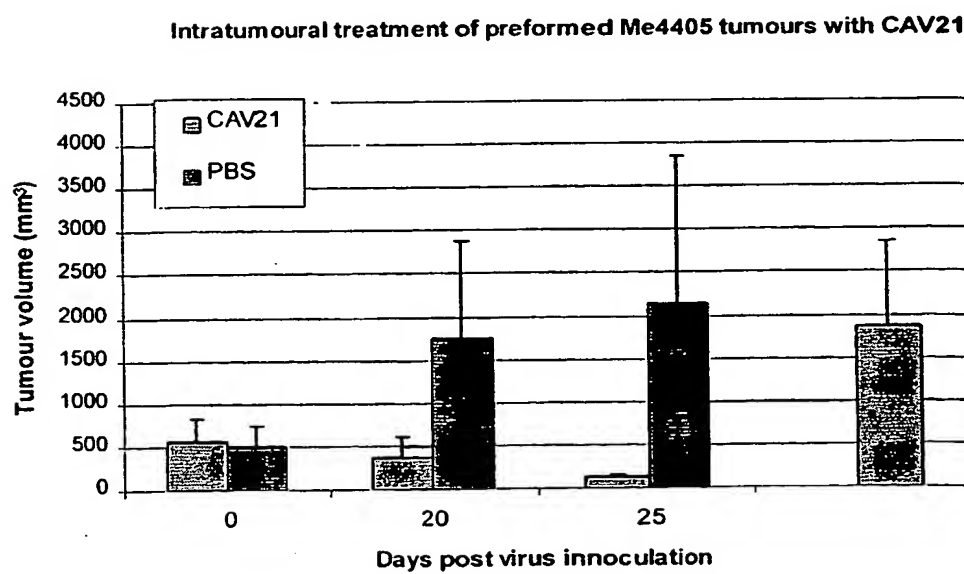


Figure 15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU00/01461

A. CLASSIFICATION OF SUBJECT MATTER		
Int. Cl. ⁷ : A61K 39/12, 39/125, 39/235, 39/245, 39/215 A61P 35/00, 35/04		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) A61K WITH ELECTRONIC DATABASE SEARCH TERMS AS INDICATED BELOW		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC AS ABOVE		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) DERWENT/CAS/MEDLINE: melonoma and apoptosis or cell death or lysis and enterovirus or coxsackievirus or ICAM or CAM or VCAM or DAF or CD55.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Investig. New Drugs, Vol 17 (1999) pages 375-386, "Oncolytic Viruses", Neumunaitis, J - whole document	1, 6, 7, 18, 19, 21, 25, 33, 38, 39, 45, 46, 48, 55, 56, 62, 67-69, 78- 105, 110, 111, 117, 118, 119, 121,
Y		2-5, 8-17, 20, 22-24, 26-32, 34-37, 40-44, 47, 49-54, 57-61, 63- 66, 69-77, 106- 109, 112-116 120, 122-129
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input type="checkbox"/> See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 22 January 2001		Date of mailing of the international search report 25 January 2001
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929		Authorized officer JENNIFER FERNANCE Telephone No : (02) 6283 2416

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU00/01461

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. Virology, 72/11 (1998) pages 9407-9412, "Viral Cell Entry Induced by Cross-linked Decay-Accelerating Factor", Shafren	1-17,33-44, 55-66, 78-117
Y	- whole document	18,-32, 45-54, 67-77, 118-129
Y	J. Clin. Invest, 81/4 (1988) pages 1122-1128, "Decay-Accelerating Factor Protects Human Tumor Cells from Complement-mediated Cytotoxicity in Vitro", Cheung et al	1-129
Y	- whole document	
Y	Clin. Exp. Metastasis, Vol 8 Abstract 80 (1990) page 48, "Intercellular adhesion molecule-1 (ICAM-1) expression by human melanoma cells: association with leukocyte aggregation and metastatic potential", Miller et al	1-129
Y	- whole document	
Y	Canc. Immunol. Immunother., Vol 42 (1996) pages 185-192, "Characterisation of the complement-regulatory proteins decay accelerating factor (DAF, CD55) and membrane cofactor protein (MCP, CD46) on a human colonic adenocarcinoma cell line", Bjorg et al	1-129
X	- whole document	
X	WO-A-99/28750 (University North Carolina at Chapel Hill) 10 June 1999	1, 6, 7, 18, 19, 21, 25, 33, 38, 39, 45, 46, 48, 55, 56, 62, 67-69, 78-105, 110, 111, 117, 118, 119, 121,
Y	- whole document	
Y		2-5, 8-17, 20, 22-24, 26-32, 34-37, 40-44, 47, 49-54, 57-61, 63-66, 69-77, 106-109, 112-116 120, 122-129
X	Human Gene Therapy, 9/16 (1998) pages 2363-2373, "The Presence of Human Cocksackievirus and Adenovirus Receptor Is Associated with Efficient Adenovirus-Mediated Transgene Expression in Human Melanoma Cell Cultures", Hemmi et al	1, 6, 7, 18, 19, 21, 25, 33, 38, 39, 45, 46, 48, 55, 56, 62, 67-69, 78-105, 110, 111, 117, 118, 119, 121,
Y	- whole document	
Y		2-5, 8-17, 20, 22-24, 26-32, 34-37, 40-44, 47, 49-54, 57-61, 63-66, 69-77, 106-109, 112-116 120, 122-129
Y	Cancer Biology, 7/3 (1996) pages 129-138, "The role of α v-integrins in tumour progression and metastasis", Marshall et al	18, 21, 29-32, 45, 48, 67, 68, 118, 121,
Y	- whole document	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01461

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Fundamental Virology, 3 rd Edn (1996), Fields et al pages 25, 174-176, 499-500	1-129
PX	Derwent Abst. Acc. No: 2000-549414/50 class BO4, D16 WO 200050618 A (Japanese Found Cancer Res) 31 August 2000	1, 6, 7, 18, 19, 21, 25, 33, 38, 39, 45, 46, 48, 55, 56, 62, 67-69, 78-105, 110, 111, 117, 118, 119, 121,
PY		2-5, 8-17, 20, 22-24, 26-32, 34-37, 40-44, 47, 49-54, 57-61, 63-66, 69-77, 106-109, 112-116 120, 122-129